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Hyperglycaemia-induced chemo-resistance in breast cancer cells: role of the estrogen receptor.

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Abstract

Breast cancer patients with diabetes respond less well to chemotherapy; in keeping with this we determined previously that hyperglycaemia induced chemo-resistance in estrogen receptor (ER α) positive breast cancer cells and showed that this was mediated by fatty acid synthase (FASN). More recent evidence suggests that the effect of metabolic syndrome and diabetes is not the same for all subtypes of breast cancer with inferior disease-free survival and worse overall survival only found in women with ER α positive breast cancer and not for other subtypes. Here we examined the impact of hyperglycaemia on ER α negative breast cancer cells and further investigated the mechanism underlying chemo-resistance in ER α with a view to identifying strategies to alleviate hyperglycaemia-induced chemo-resistance. We found that hyperglycaemia-induced chemo-resistance was only observed in ER α breast cancer cells and was dependent upon the expression of ER α as chemo-resistance was negated when the ER α was silenced. Hyperglycaemia induced an increase in activation and nuclear localisation of the ER α that was downstream of FASN and dependent on the activation of mitogen activated protein kinase (MAPK). We found that Fulvestrant successfully negated the hyperglycaemia-induced chemo-resistance, whereas Tamoxifen had no effect. In summary our data suggests that the ER α may be a predictive marker of poor response to chemotherapy in breast cancer patients with diabetes. It further indicates that anti-estrogens could be an effective adjuvant to chemotherapy in such patients and indicates the importance for the personalised management of breast cancer patients with diabetes highlighting the need for clinical trials of tailored chemotherapy for diabetic patients diagnosed with ER α positive breast cancers.

58 Introduction

59 Breast cancer is the most common malignancy in women with a lifetime risk of 1 in 8. In all
60 Western societies women who present with breast cancer are increasingly likely to also suffer
61 from co-morbid conditions such as diabetes and obesity due to the increasingly high prevalence
62 of these conditions in the general population. In a study of over a thousand women treated for
63 breast cancer at MD Anderson Cancer Centre in Houston, 30% were found to be obese and a
64 further 32% overweight (Litton, et al. 2008). In addition the prevalence of metabolic syndrome in
65 patients with breast cancer has been reported to be between 39-50% (Healy, et al. 2010;
66 Stebbing, et al. 2012).

67 For all cancers, the co-morbidity of type 2 diabetes has been reported to be associated with poor
68 prognosis and reduced survival: with a mean survival period of 10.4 years for those with diabetes
69 and 14.3 years for those without (Currie, et al. 2012). In women with breast cancer, having
70 metabolic syndrome was also associated with more aggressive tumour characteristics (Healy et
71 al. 2010) and being obese confers worse overall survival (Litton et al. 2008).

72 Evidence also suggests that the effect of metabolic syndrome and diabetes is not the same for all
73 subtypes of breast cancer. In a study of women involved in three large trials, inferior disease-free
74 survival and worse overall survival was only identified in women with estrogen receptor (ER α)
75 positive breast cancer and not for other subtypes (Sparano, et al. 2012). A recent study found
76 that hyperglycaemia was the only feature associated with metabolic syndrome that was
77 associated with disease progression following chemotherapy in a cohort of women with breast
78 cancer (73% of whom had ER α positive tumors) (Stebbing et al. 2012).

79 In an attempt to identify the mechanisms underlying these in vivo clinical observations we
80 previously investigated the impact of raised glucose levels on chemo-sensitivity of ER α positive
81 breast cancer cells. We found that hyperglycaemia induced chemo-resistance in these cells, but
82 not in non-malignant breast epithelial cells, and showed that this was mediated by increased

activity of the enzyme fatty acid synthase(FASN) which synthesises fatty acids (with glucose as the main substrate) (Zeng, et al. 2010). In keeping with the current clinical data (Stebbing et al. 2012), we found that hyperglycaemia-induced chemo-resistance was observed in ER α positive but not in ER α negative breast cancer cells. We further determined that a functional ER α was required to mediate the hyperglycaemia-induced chemo-resistance and finally that anti-estrogens may be an effective adjuvant to chemotherapy in breast cancer patients with diabetes.

91 **Materials and Methods**

92 **Reagents and antibodies**

93 All chemicals were purchased from Sigma (Poole, Dorset, UK). All siRNAs and the transfection
94 reagent, HiPerFect were purchased from Qiagen (Crawley, W. Sussex, UK).

95 **Cell Culture**

96 The human breast cancer cell lines MCF7, T47D, MDA-MB-231 and Hs578T were purchased from
97 ATCC that authenticates using short tandem repeat (STR) DNA profiles and the cells were used for
98 a maximum of 10 passages. They were maintained as described before (McIntosh, et al. 2010;
99 Zeng et al. 2010).

100 **Dosing protocols**

101 Cells were seeded in normal (5mM) glucose-containing growth media for 24 hours and then
102 switched to either high (25mM) a or normal (5mM) glucose-containing serum free media for a
103 further 48 hours with or without a MAPK inhibitor, UO126 (30µm) or for 24 hours prior to dosing
104 with chemotherapy drugs doxorubicin (0-40µM), paclitaxel (0-300µM) or C2-ceramide (0-30µM)
105 in the presence or absence of target siRNA to the ERα, fatty acid synthase (FASN) or non-silencing
106 (ns) siRNA (as described previously (Foulstone, et al. 2013; Zeng et al. 2010)) or, Tamoxifen (1µM)
107 or Fulvestrant (100nM). We used two siRNAs to silence both ERα and FASN: the second siRNA we
108 used in this study for FASN is illustrated in supplementary figure 1.

109 **Cell viability**

110 This was determined by trypan blue dye exclusion assay as outlined before (McIntosh et al. 2010;
111 Zeng et al. 2010). We confirmed apoptotic cell death by assessing the cleavage of poly (ADP-
112 ribose) polymerase (PARP) using Western immunoblotting as described previously (Thomas, et al.
113 2009).

114

115

Cell Fractionation Assay

Cytoplasmic and nuclear fractions were separated with NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Cat#78835), following the manufacturers' instructions. Protein content of fractionated proteins and whole cell lysates were assessed using a BCA protein assay reagent kit (Pierce: Rockford, IL, USA) and then run on either 8/12% SDS-PAGE and transferred to a Hybond-C nitrocellulose membrane (GE Healthcare, Bucks, UK) as previously described (Zeng et al. 2010). Membranes were probed with p-ER(1:500), p-MAPK (1:1000), MAPK (1:500), tubulin (1:5000), lamin (1:1000), GAPDH (1:5000), β -actin (1:10000), FASN (1:1000) and ER α (1:750), PARP (1:1000), following the manufacturers' instructions. Tubulin and lamin were used as markers for identifying cytoplasmic and nuclear cell fractions respectively. Secondary antibodies conjugated to peroxidase were used: anti-mouse for p-ER α (1:1500), tubulin (1:5000), lamin (1:2000), GAPDH (1:5000), FASN (1:5000), ER α (1:1500), PARP (1:2000) and β -actin (1:10000). Chemiluminescence was detected using the Chemi-Doc-IT Imaging (UVP, Biorad) and analysed using Vision Works Analysis Software (UVP Inc., Upland, Ca, USA).

Chromatin Immunoprecipitation assay (ChIP)

ChIP was performed using the Imprint Immunoprecipitation kit (Cat# CHP1) from Sigma Aldrich as described before (Biernacka, 2013). Briefly, after DNA-protein crosslinking with formaldehyde, samples were sonicated and immunoprecipitated with a ChIP grade anti-ER α antibody (Millipore, Cat# 17-603). Anti-RNA polymerase II and mouse IgG supplied with the kit were used as positive and negative controls respectively. 5% input DNA was used for quantification. PCR (HotStarTaq Plus PCR Kit from Qiagen) was performed with purified DNA. The following primers were used for Cyclin D1 (CCND1): forward (-1039) AACAAAACCAATTAGGAACCTT, reverse (-770) ATTCCTTCATCTTGTCCTTCT (as reported in Zheng, 2013(Zheng, et al. 2013)) After 38cycles, PCR products were detected by 2% agarose gel electrophoresis, stained with midori Green (Nippon

140 Genetics, # MG04) and analysed by Bio-Plex Imaging system from Bio-Rad (Serial No.
141 731BR01508).

142 **3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) ASSAY**

143 The MTT assay was performed as described previously (Gill, et al. 1997; Perks, et al. 2003) .
144 Briefly, cells were seeded into 96-well plates at 7500 (MCF7) or 22500 (T47D and MDA-MB-231)
145 cells and treated with the chemotherapeutic drugs, doxorubicin, paclitaxel and ceramide for 48
146 hrs. Cells were incubated with 7.5mg/ml MTT solution for 3 hrs at 37°C. The reaction was
147 stopped by the addition of 50µl stop solution (0.1M HCL+ 10% Triton-100) at room temperature
148 for 20 minutes. The absorbance at 590nm was measured using an ELISA plate reader.

149

150 **[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium** 151 **(MTS) Assay**

152 Cells were incubated with an MTS (2mg/ml, Promega, cat# G1118)/PMS (0.92mg/ml, Sigma
153 P9625) solution at a ratio of 20:1 for 3 hrs at 37°C. 200µl of cell medium was used to measure the
154 absorbance at 490nm with an ELISA plate reader. The cells remain viable for further analysis
155 using this assay as opposed to the MTT assay and so we used the MTS assay for experiments in
156 which we also wished to assess cell number and viability.

157

158 **Statistical Analysis**

159 Data were analysed with SPSS 12.0.1 for Windows using one-way ANOVA followed by least
160 significant difference (LSD) post-hoc test. A statistically significant difference was considered to
161 be present at $P < 0.05$.

162

163

Results

Hyperglycaemia-induced chemo-resistance is only observed in ER α positive breast cancer cells.

We demonstrated previously that ER α positive breast cancer cells, MCF7 and T47D, were resistant to cell death induced by chemotherapeutics (doxorubicin, paclitaxel and ceramide) when exposed to high levels of glucose (Zeng et al. 2010). With MCF7 cells Fig 1A confirms these previous data and Fig 1B also shows that the MTT assay also indicates the differential effects induced by altered levels of glucose: increasing doses of C2 reduced metabolic activity more effectively in normal compared to high glucose conditions. Using both the MTT assay and cell counting in this study we found that in contrast to the ER α positive breast cancer cells, ER α negative Hs578T (Fig 1C & D) and MDA-MB-231 (Fig 1E & Fig1F) cells exhibited no chemo-resistance following exposure to doxorubicin, paclitaxel or ceramide in high compared to normal glucose conditions. With Hs578T cells ceramide was able to induce cell death at 10 μ M and not at the lower doses as demonstrated by the induction of PARP cleavage (insert Fig 1C), this confirms that any differential effects in response to ceramide in relation to the levels of glucose at the lower doses of ceramide as observed in the dose response (Fig 1C) were not related to the induction of cell death. We characterised the growth of the cells when exposed to normal and high levels of glucose and found that basal cell growth of ER α positive and negative cells was unaffected over 48 hours by changes in the levels of glucose (supplementary Fig2: A-D). Having observed chemoresistance in high glucose in the ER α positive breast cancer cells, we chose MCF-7 cells and assessed changes in glucose uptake and expression of the key glucose transporters, GLUTs 1 and 12. We found that there was a 1.1-fold increase in glucose uptake in 25mM glucose that was associated with an increase in expression of GLUT 1 ($p<0.05$) and GLUT 12 ($p<0.05$). Clearly other glucose transporters are likely to play a role (supplementary Fig3 A-C).

Silencing the ER α alleviates chemo-resistance in hyperglycaemic conditions in ER α positive breast cancer cells.

Having observed that hyperglycaemia only induced chemo-resistance in ER α positive breast cancer cells, we next investigated if the presence of the ER α was required. With MCF7 cells, we found that in the presence of the ns siRNA, hyperglycaemic conditions reduced the ability of doxorubicin to induce cell death compared to euglycaemic conditions (from 31% to 23%; $p=0.05$) (Fig 2A) whereas with the ER α silenced, the hyperglycaemia-induced chemo-resistance was negated (Fig.2A). Similarly with T47D cells (Fig2B), in the presence of the ns siRNA, hyperglycaemic conditions reduced the ability of doxorubicin to increase cell death compared to euglycaemic conditions (from 24% to 18%; $p<0.05$) (Fig 2B) whereas with the ER α silenced, the hyperglycaemia-induced chemo-resistance was negated (Fig. 2B). With MCF7 and T47D cells we also showed that in the presence of the ns siRNA that high glucose reduced the ability of doxorubicin to decrease metabolic activity (from 0.43 fold to 0.26 fold and from 0.37 fold to 0.06; $p=0.05$; $p<0.05$ respectively) (Figs 2C & 2D) and that this was negated in each cell line when the ER α was silenced (Figs 2C & 2D respectively).

The western blot (Fig 2E) shows effective silencing of the ER α in both MCF7 and T47D cells in 5 and 25mM glucose conditions. The blot for PARP also confirms the chemo-resistant effect of high glucose by showing a clear reduction in doxorubicin-induced PARP cleavage in the ns hyperglycaemic compared to the ns euglycaemic conditions with both cell lines.

Hyperglycaemia increases phosphorylation and nuclear localization of ER α

Using cellular fractionation followed by Western blotting, we examined alterations in the localisation and phosphorylation of ER α . With MCF7 cells (Fig 3A & B), following exposure to high levels of glucose, we observed a shift in the localisation of the ER α with the ratio of ER α in cytoplasmic and nuclear part reduced from 1.9 to 0.8 ($p<0.05$). As ER α nuclear localisation is concomitant with an increase in ER α phosphorylation, we assessed changes in one of the key ER α

phosphorylation sites: ser¹¹⁸. We observed that high levels of glucose increased total levels of ERα ser¹¹⁸ phosphorylation and that as anticipated there was a 1.6 fold increase in ERα ser¹¹⁸ phosphorylation in the nucleus (p<0.01). Similarly with T47D cells (Fig 3A & B) we observed a shift in the localisation of the ERα with the ratio of ERα in cytoplasmic and nuclear part reduced from 1.4 to 1.1 (P<0.05) concomitant with a 1.4 fold increase ERα ser¹¹⁸ phosphorylation in the nucleus (p<0.05).

Hyperglycaemia increases nuclear ERα binding to a target gene, cyclin D1 (CCND1)

As a further confirmation of the increased nuclear localisation of the ERα, we chose to assess alterations in the association of ERα with one of its known target genes, cyclin D1, CCND1. Using ChIP assay we found that high levels of glucose caused a 2.7 fold increase (p<0.05) with MCF7 cells and an 11.7 fold increase (p<0.05) with T47D cells (Fig 3C & D) in the association of ERα with the CCND1 gene.

The hyperglycaemia-induced increase in ERα ser¹¹⁸ phosphorylation is downstream of fatty acid synthase (FASN)

We had shown previously in ERα positive breast cancer cells that hyperglycaemia-induced chemo-resistance was dependent upon FASN (Zeng et al. 2010). Having now demonstrated a role for the ERα in hyperglycaemia-induced chemo-resistance, we silenced FASN using siRNA in both MCF7 (Fig 4A) and T47D (Fig 4B) cells to determine any impact on the ERα and levels of ERα ser¹¹⁸ phosphorylation. We found that silencing FASN reduced levels of the ERα in both normal and high glucose and reduced the ability of hyperglycaemia to activate ERα ser¹¹⁸ phosphorylation (Fig 4C) suggesting that FASN is acting upstream of the ERα.

238 **FASN regulates p-ER α (ser118) levels via the MAPK pathway**

239 Having observed that the effect of hyperglycaemia on the phosphorylation of the ER α was
240 downstream of FASN, we next wished to elucidate the mechanism through which FASN regulated
241 p-ER α ser¹¹⁸ levels. With MCF-7 cells, we found that in the presence of the non-silencing FASN
242 siRNA, hyperglycaemic conditions increased p-MAPK levels (x 1.8 fold increase; p=0.01) compared
243 to euglycaemic conditions and that this was negated when FASN was silenced (Fig 5A & B). To
244 determine whether MAPK signalling pathway was required for the hyperglycaemia-induced
245 increase in ER α ser¹¹⁸ phosphorylation, we used U0126, a MAPK inhibitor. As illustrated in Fig 5C
246 and D, treatment with U0126 completely blocked the ability of hyperglycaemia to activate ER α
247 ser¹¹⁸ phosphorylation. Taken together, these results indicate that FASN regulates p-ER α
248 (ser118) levels via activation of MAPK.

249 **Fulvestrant blocks but Tamoxifen has no effect on hyperglycaemia-induced chemo-resistance**

250 Having shown that silencing the ER α using siRNA negated chemo-resistance induced by high
251 glucose, we then assessed the effects of blocking the estrogen receptor in a more clinically
252 relevant manner by using two anti-estrogens, Fulvestrant (selective estrogen receptor down
253 regulator, SERD) and Tamoxifen (selective estrogen receptor modulator, SERM). We first ensured
254 we were using effective doses of each of the drugs.

255 With fig 6A & B and Fig 7A & B we show that Fulvestrant and Tamoxifen (respectively) effectively
256 blocked estrogen-induced ER α ser¹¹⁸ phosphorylation in both MCF7 and T47D cell lines. We also
257 confirmed in both cell lines that Fulvestrant down-regulates the ER α and Tamoxifen stabilises it as
258 reported in the literature (supplementary fig 13 B&C). Having identified effective doses of both
259 drugs we investigated if either Fulvestrant or Tamoxifen would negate hyperglycaemia-induced
260 chemo-resistance as we had observed when the estrogen receptor was artificially silenced using
261 siRNA. Fig 6C & 6D indicate that hyperglycaemia-induced resistance to doxorubicin-induced cell

death (18 % to 11% $p < 0.01$ and 13% to 7% respectively) was blocked by Fulvestrant in both MCF7 and T47D breast cancer cells. In contrast Fig 7C (MCF7) & D (T47D) show that Tamoxifen was ineffective in negating the hyperglycaemia-induced chemo-resistance in either cell line.

281 Discussion

282 The current report has determined that hyperglycaemia-induced chemo-resistance only occurs in
283 breast cancer cell lines that possess a functional ER α and has identified that the ER α is key in
284 mediating this hyperglycaemia-induced chemo-resistance. We believe that our novel data may
285 explain important clinical observations: in a study of women involved in three large trials, inferior
286 disease-free and overall survival was only found in women with ER positive breast cancer and not
287 for other subtypes(Sparano et al. 2012). In addition, that hyperglycaemia was the only feature
288 associated with metabolic syndrome that was linked with disease progression following
289 chemotherapy in a cohort of women with breast cancer (73% of whom had ER positive
290 tumors)(Stebbing et al. 2012). It has been reported that estrogen receptors are important
291 regulators of components of the glycolytic pathway and contribute to the Warburg effect in
292 cancer cells(Cai, et al. 2012). Our data indicate that the ER α is also an important determinant of
293 how metabolic conditions specifically affect breast cancer cells and their response to
294 chemotherapy. We clearly observed that silencing the ER α in ER α positive breast cancer cells
295 negated hyperglycaemia-induced chemo-resistance; consistent with these findings we
296 determined that exposing ER α negative breast cancer cells to different levels of glucose did not
297 influence chemo-sensitivity. The ER α has been linked to chemo-resistance previously: Tokuda et
298 al showed that silencing the ER α in MCF7 breast cancer cells enhanced sensitivity to paclitaxel
299 (Tokuda, et al. 2012). This study was only performed under hyperglycaemic conditions and in that
300 context their results were consistent with our findings.

301 As the ER α can be located at numerous sites within the cell we assessed if exposure to high
302 glucose impacted on the localisation of the ER α . We found that hyperglycaemia increased the
303 relative amounts of ER α in the nucleus compared to the cytoplasm. The ER α needs to be
304 phosphorylated to translocate to the nucleus: in keeping with the ER α localisation data we
305 observed a significant increase in phosphorylation of ER α at ser¹¹⁸. To corroborate these findings

we assessed the level of ER α binding to one of its known target genes, cyclin D1 when exposed to different levels of glucose: as anticipated based on the localisation and phosphorylation data, we found an increase in association of the ER α with the cyclin 1 gene when ER α positive breast cancer cells were exposed to high levels of glucose. Ross-Innes et al assessed the dynamics of ER α binding to DNA in clinical breast cancer samples and found that differential ER α binding was associated with clinical outcome in breast cancer: ER α bound to different sites and with different affinities depending on the stage (Ross-Innes, et al. 2012). In light of these novel findings our data may suggest that breast cancer patients with altered metabolism may have tumour cells with altered ER α /DNA binding patterns that may contribute to chemo-resistance.

We showed previously that hyperglycaemia-induced chemo-resistance was dependent upon fatty acid synthase (FASN) (Zeng et al. 2010). Associations between the ER α and FASN have been identified in breast cancer cells previously (Lupu and Menendez 2006). We delineated that our new data, showing the important role of the ER α , was related to our previously defined signalling pathway. Our data suggests that the effect of hyperglycaemia on the phosphorylation of the ER α is downstream of FASN as silencing FASN reduced activation of the ER α : in addition that the increased phosphorylation of the ER α was mediated by MAPK activation downstream of FASN.

We then blocked the estrogen receptor in a more clinically relevant manner using two anti-estrogens, Fulvestrant and Tamoxifen to assess their effectiveness in alleviating the resistance induced by hyperglycaemic conditions. We found that Fulvestrant successfully negated the hyperglycaemia-induced chemo-resistance, whereas Tamoxifen had no effect. We believe this relates to how these two drugs act: Fulvestrant degrades the ER α whereas Tamoxifen stabilises the protein. The mechanism by which Fulvestrant acts would be most comparable to our experiments where we artificially silenced the ER α with siRNA.

In summary our data provides a mechanism to support the clinical studies indicating that women with ER α positive breast cancer who also have diabetes respond less well to chemotherapy: it suggests that the ER α may be a predictive marker of poor response to chemotherapy in breast

cancer patients with diabetes. It further indicates that certain classes of anti-estrogen therapy may prove effective adjuvants to chemotherapy in such patients but the specific type of anti-estrogen needs to be considered carefully. Our data indicates the potential importance and benefit of personalised medical therapy in the management of breast cancer, highlighting the need for clinical trials of tailored chemotherapy for diabetic patients diagnosed with ER α positive breast cancers.

Declaration of interest, Funding and Acknowledgements

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415

Figure Legends

Figure 1: Hyperglycaemia-induced chemo-resistance is only observed in ER α positive breast cancer cells. **A.** Using trypan blue dye exclusion method, the percentage cell death was assessed in the ER α positive MCF7 cells treated with chemotherapeutics (Doxorubicin 1 μ M $p \leq 0.01$, Paclitaxel 1 μ M $p = 0.01$ and Ceramide 12 μ M $p = 0.01$) in 5mM and 25mM glucose for 24 hours ($n = 3$ experiments). Changes in metabolic activity of the MCF-7 cells in 5mM and 25mM glucose in response to ceramide (0- 20 μ M) was examined using an MTT assay (**B**) ($n = 3$ experiments, *: $p \leq 0.05$). The metabolic activity of the ER α negative Hs578T cells in 5mM and 25mM glucose in response to doxorubicin (DOX, 0- 20 μ M), ceramide (C2, 0-30 μ M) and paclitaxel (Pac, 0-300 μ M) was examined with MTT assay (**C**) ($n = 3$ experiments). Insert shows ceramide-induced PARP cleavage (85kD fragment) by Western blotting ($n = 3$ experiments). Percentage of cell death triggered by these drugs in 5mM and 25mM glucose was assessed by trypan blue dye exclusion assay (**D**) ($n = 3$ experiments). The change in metabolic activity of another ER α negative cell line, MDA-MB-231 in 5mM and 25mM glucose exposed to the above drugs is shown in (**E**) and percentage of cell death in (**F**) ($n = 3$ experiments).

Figure 2: Silencing the ER α alleviates chemo-resistance in hyperglycaemic conditions in ER α positive breast cancer cells, compared to the non-silencing siRNA control (ns). Percentage of cell death induced by doxorubicin (DOX, 1 μ M in MCF7 and 5 μ M in T47D cells) was assessed in MCF7 (**A**) or T47D (**B**) cells with or without ER α knocked down with 20nM siRNA in 5 or 25mM glucose ($n = 3$ experiments). Changes of metabolic activity induced by doxorubicin (DOX) in MCF7 (**C**) or T47D (**D**) cells were also measured with or without ER α silencing in 5 or 25mM glucose ($n = 3$ experiments). Effective ER α knocking down and PARP cleavage was shown in (**E**). β -actin probing was used as a loading control ($n = 3$ experiments).

Figure 3: Hyperglycaemia increases phosphorylation and nuclear localization of ER α and increases nuclear ER α binding to a target gene, cyclin D1 (CCND1). Cell fractionation and Western blotting

were performed to examine cytoplasmic or nuclear location of ER α and p-ER α (ser¹¹⁸) in MCF7 and T47D cells **(A)** under 5 or 25mM glucose condition. Tubulin and Lamin A/C blots were used as markers for cytoplasmic and nuclear compartments respectively (n=3 experiments,). The densitometry measurements from the western blot are shown in **(B)**, p<0.05). Chromatin Immunoprecipitation assay (ChIP) was used to examine the changes in association of the ER α with one of its target genes, cyclin D1 (CCND1) **(C)**. 5% input was used as a quantification control (n=3 experiments). The relative enrichment of the CCND1 gene bound to ER α was quantified in **(D)**, p<0.05).

Figure 4: The hyperglycemia-induced increase in ER α (ser¹¹⁸) phosphorylation is downstream of fatty acid synthase (FASN). Using western blotting, the ER α and p-ER α (ser118) abundance were assessed in MCF7 **(A)** or T47D **(B)** cells, with or without fatty acid synthase (FASN) knocked down with 20nM siRNA (n=3 experiments). β -actin was probed as a loading control. Relative fold changes of p-ER α (ser¹¹⁸) against total ER α were measured **(C)**, (p<0.05)).

Figure 5. FASN regulates p-ER α (ser118) levels via activation of MAPK. Using western blotting, the abundance of MAPK and p-MAPK were assessed in MCF-7 cells with or without FASN silenced with 20nM siRNA in 5 or 25mM glucose (n=3 experiments) **(A)**. Relative fold changes of p-MAPK against total MAPK were measured in **(B)**, p<0.05). Effect of MAPK inhibition with U0126 on p-Er α (ser118) was assessed in MCF-7 cells treated with or without U0126 (30 μ m) in 5 or 25mM glucose for 48 hours (n=3 experiments) **(C)**. The densitometry measurements from the western blot are shown in **(D)**, p<0.05).

Figure 6: Fulvestrant blocks hyperglycaemia-induced chemo-resistance. MCF7 **(A)** and T47D **(B)** cells were treated with the anti-estrogen Fulvestrant (Ful) 100nM 24 hours prior to treatment with 10nM estrogen for 20 minutes, in 5 or 25mM glucose conditions. Western blotting was performed to show

protein abundance of ER α and p-ER α (ser¹¹⁸). β -actin was probed as a loading control (n=3 experiments). Percentage of cell death induced by doxorubicin (DOX, 1 μ M in MCF7 and 5 μ M in T47D cells) was assessed in MCF7 **(C)** or T47D **(D)** cells with or without 24h hours pre-treatment of 100nM Fulvestrant in 5 or 25mM glucose (n=3 experiments).

Figure 7: Tamoxifen has no effect on hyperglycaemia-induced chemo-resistance. MCF7 **(A)** and T47D **(B)** cells were treated with the anti-estrogen Tamoxifen (TAM) 1 μ M 1 hour prior to treatment with 10nM estrogen for 20 minutes, in 5 or 25mM glucose condition. Western blotting was performed to show protein abundance of ER α and p-ER α (ser¹¹⁸). β -actin was probed as a loading control (n=3 experiments). Percentage of cell death induced by doxorubicin (DOX) was assessed in MCF7 **(C)** or T47D **(D)** cells with or without 1 hour pre-treatment of 1 μ M Tamoxifen in 5 or 25mM glucose (n=3 experiments).

Supplementary Figure 1. A second siRNA sequence was used to silence FASN in MCF7 and T47D cells under 5 or 25mM glucose condition **(A)** shows equivalent effective silencing of FASN (blot representative of 3 repeats). Western blotting showing protein abundance of ER α in MCF7 and T47D cells, treated with Fulvestrant (100nM) or Tamoxifen (1 μ M) for 24 hours, the same time length as the exposure to the chemotherapeutics **(B)**. β -actin was probed as a loading control (n=3 experiments).

Supplementary figure 2: altered levels of glucose had no effect on basal cell growth in ER α positive or negative breast cancer cell lines. MCF7, T47D (ER α positive) and Hs578T and MDA-MB-231 (ER α negative) cells were seeded in 5 mM glucose-containing growth media for 24 hours prior to being exposed to 5mM and 25 mM-glucose containing serum free media for a further 48hrs. Total cell number was assessed by cell counting. Each experiment was repeated in triplicate and performed at least 3 times.

Supplementary Figure 3 Hyperglycaemia increases glucose uptake. Glucose uptake was assessed using tritiated 2-deoxyglucose in MCF-7 cells cultured in 5 or 25mM glucose condition for 48 hours (n=3 experiments) **(A)**. Hyperglycaemia upregulates glucose transporters. The mRNA levels of GLUT1 **(B)** and GLUT12 **(C)** were quantified using SYBR green based qPCR in MCF-7 cells cultured under 5 or 25mM glucose condition for 48 hours (n=3 experiments).

Figure 1

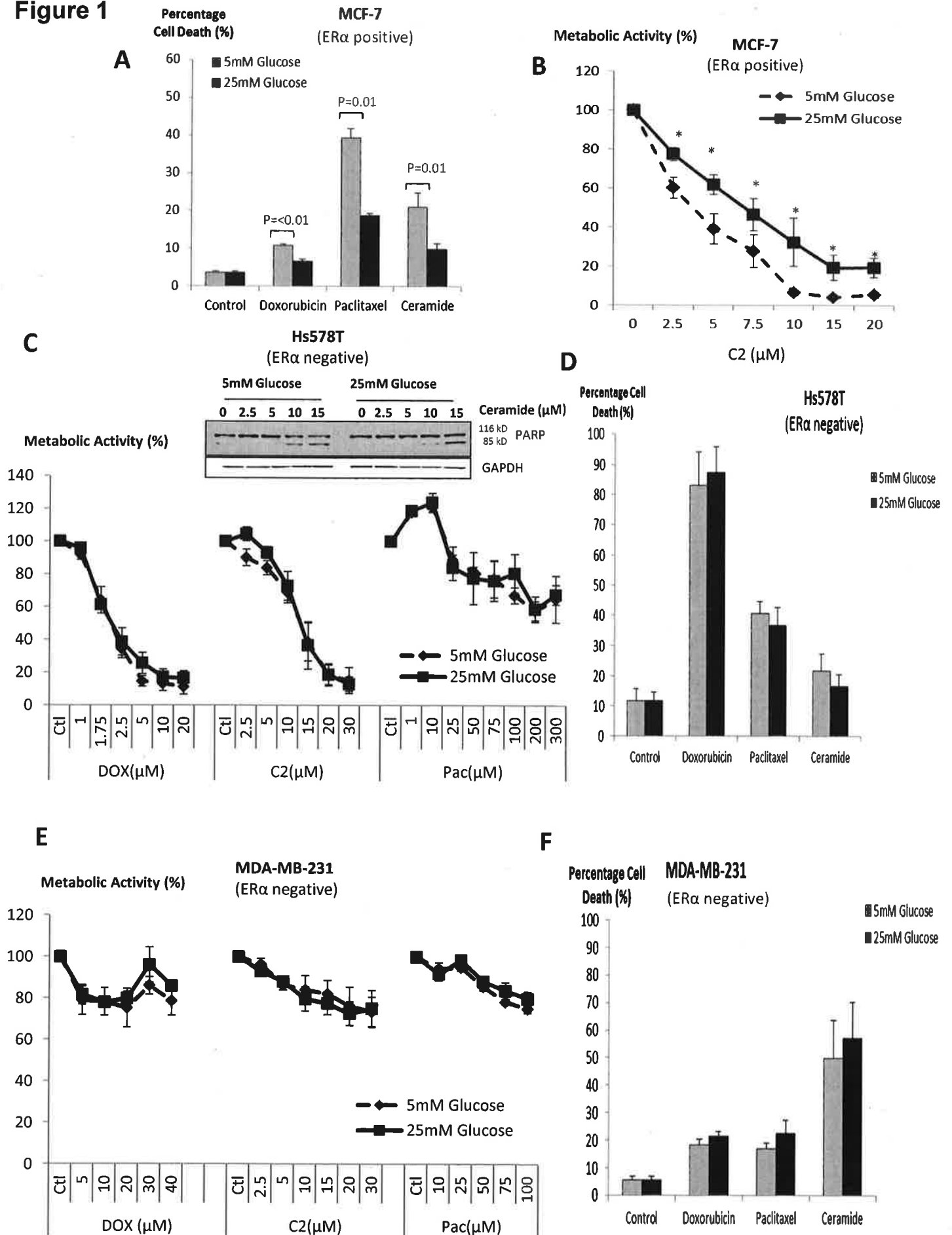


Figure 2

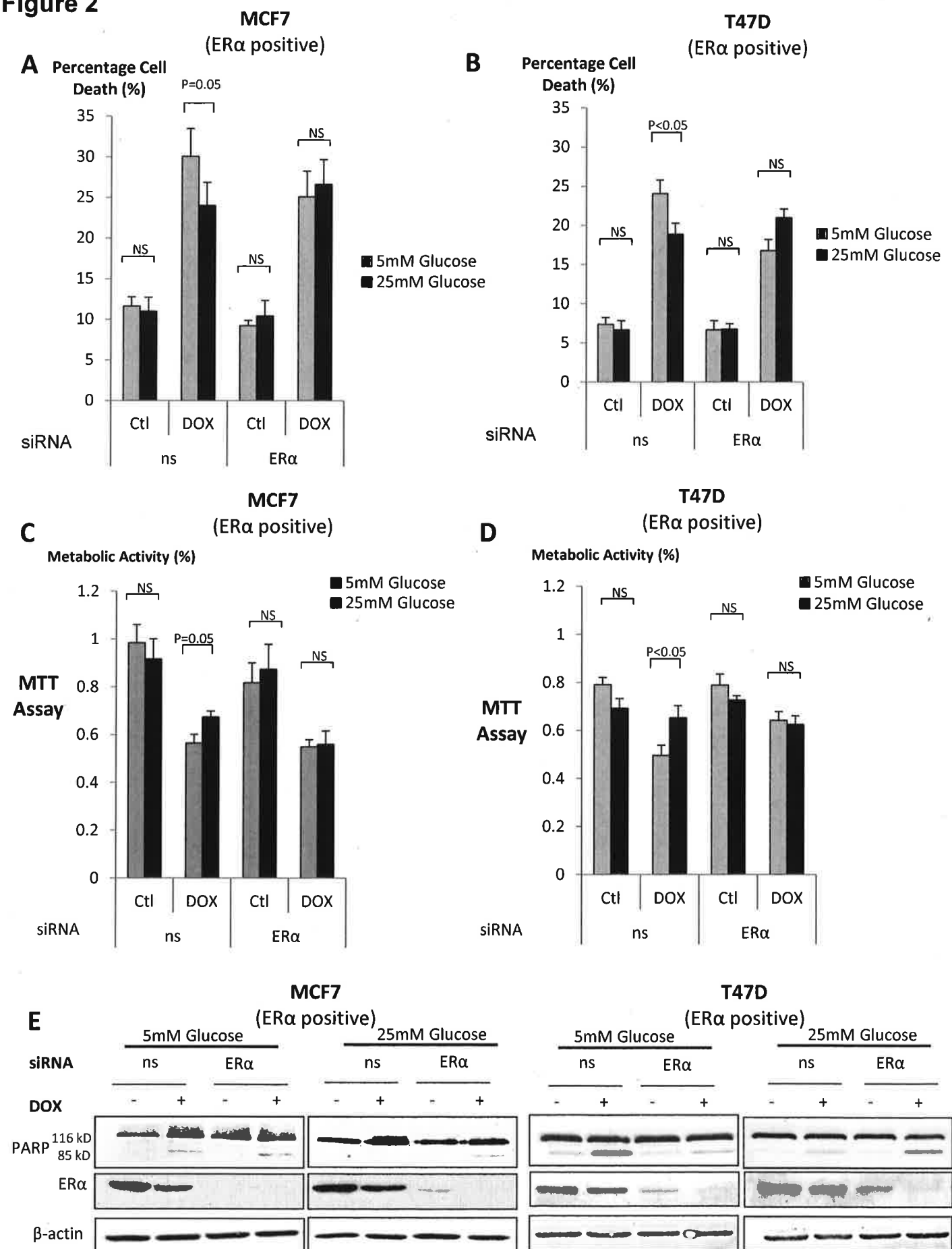
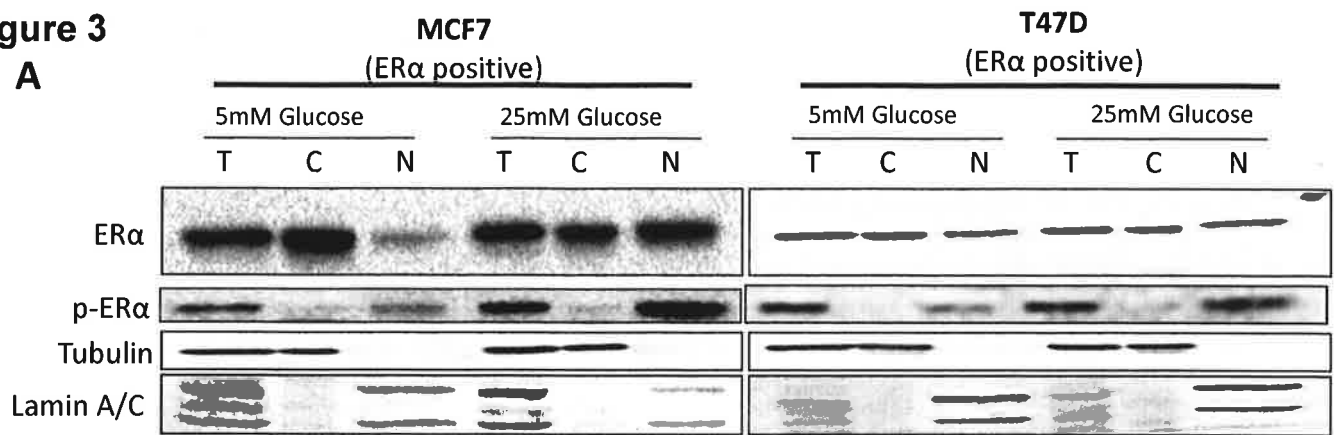


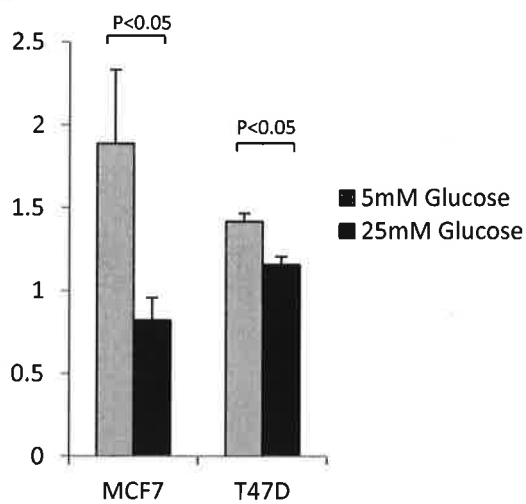
Figure 3

A

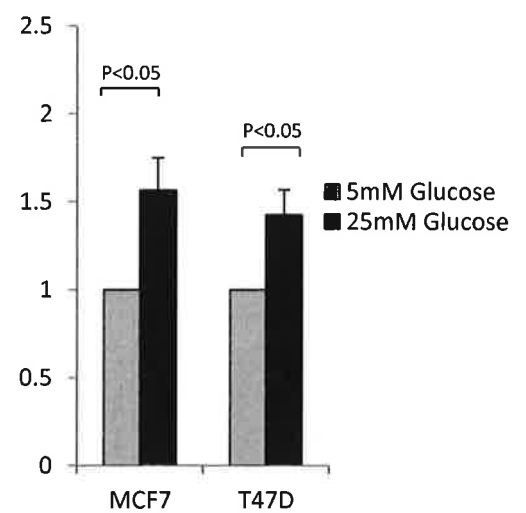


B

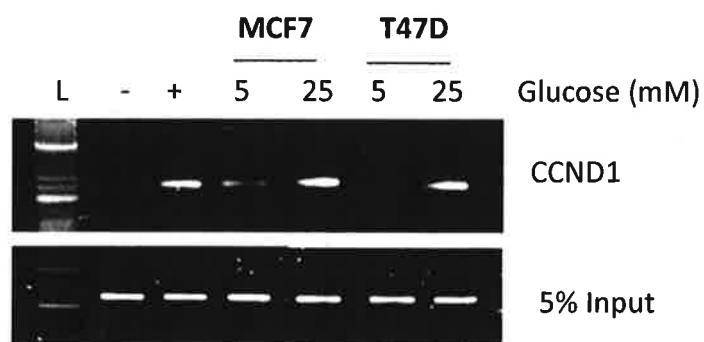
Ratio of Cytoplasmic/Nuclear
ERα Abundance



Fold Increase of p-ERα
Abundance in Nuclear



C



D

Relative enrichment against input

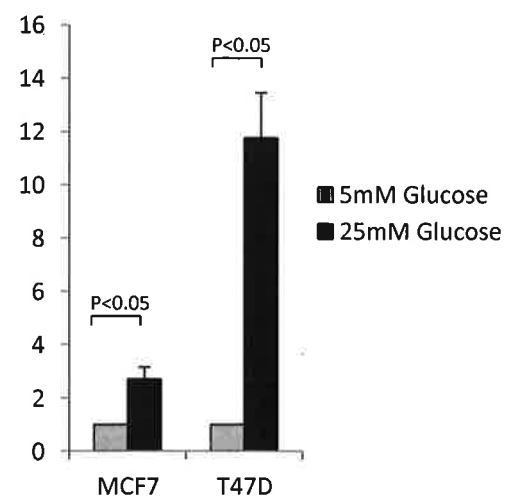


Figure 4

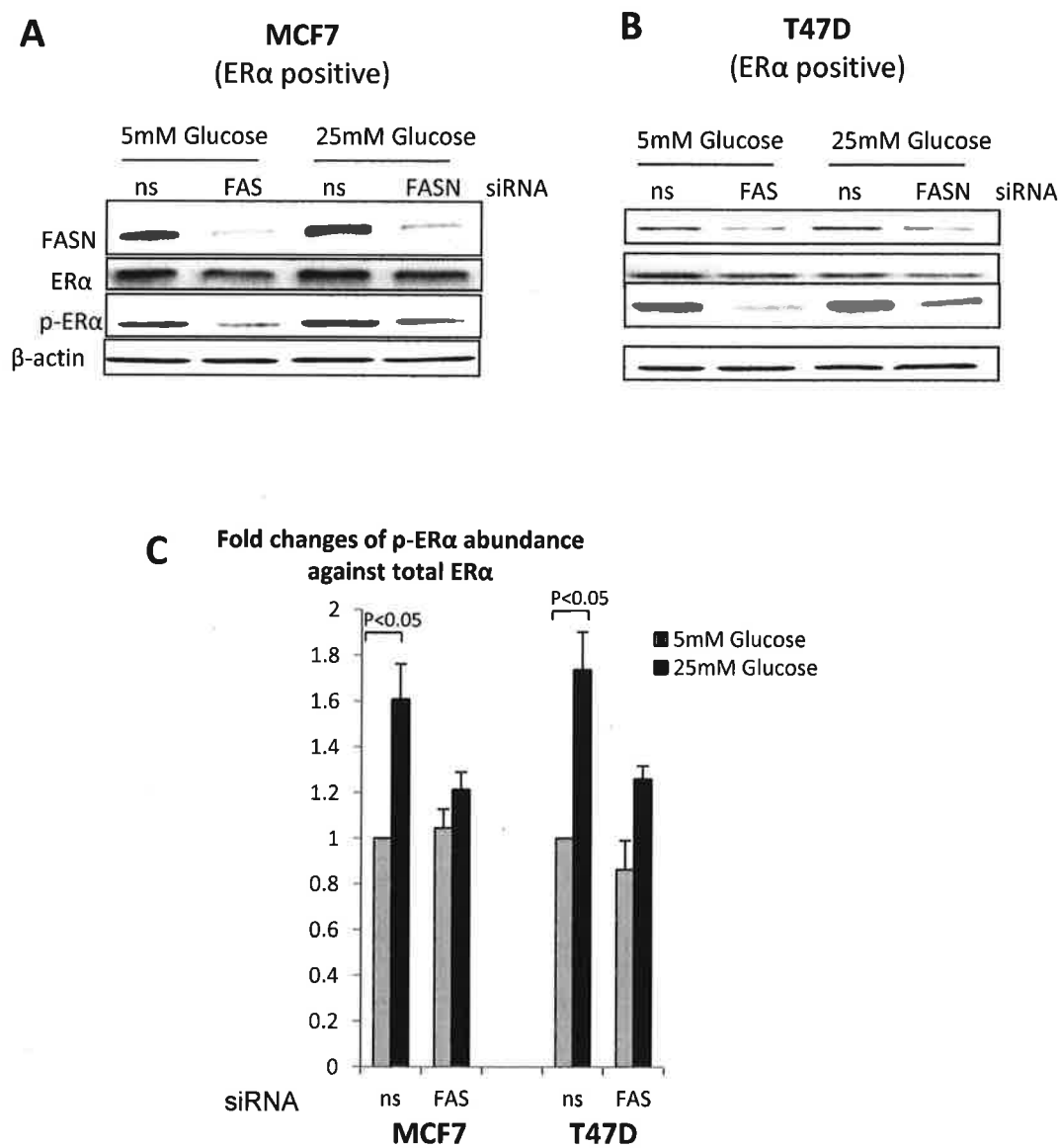


Figure 5

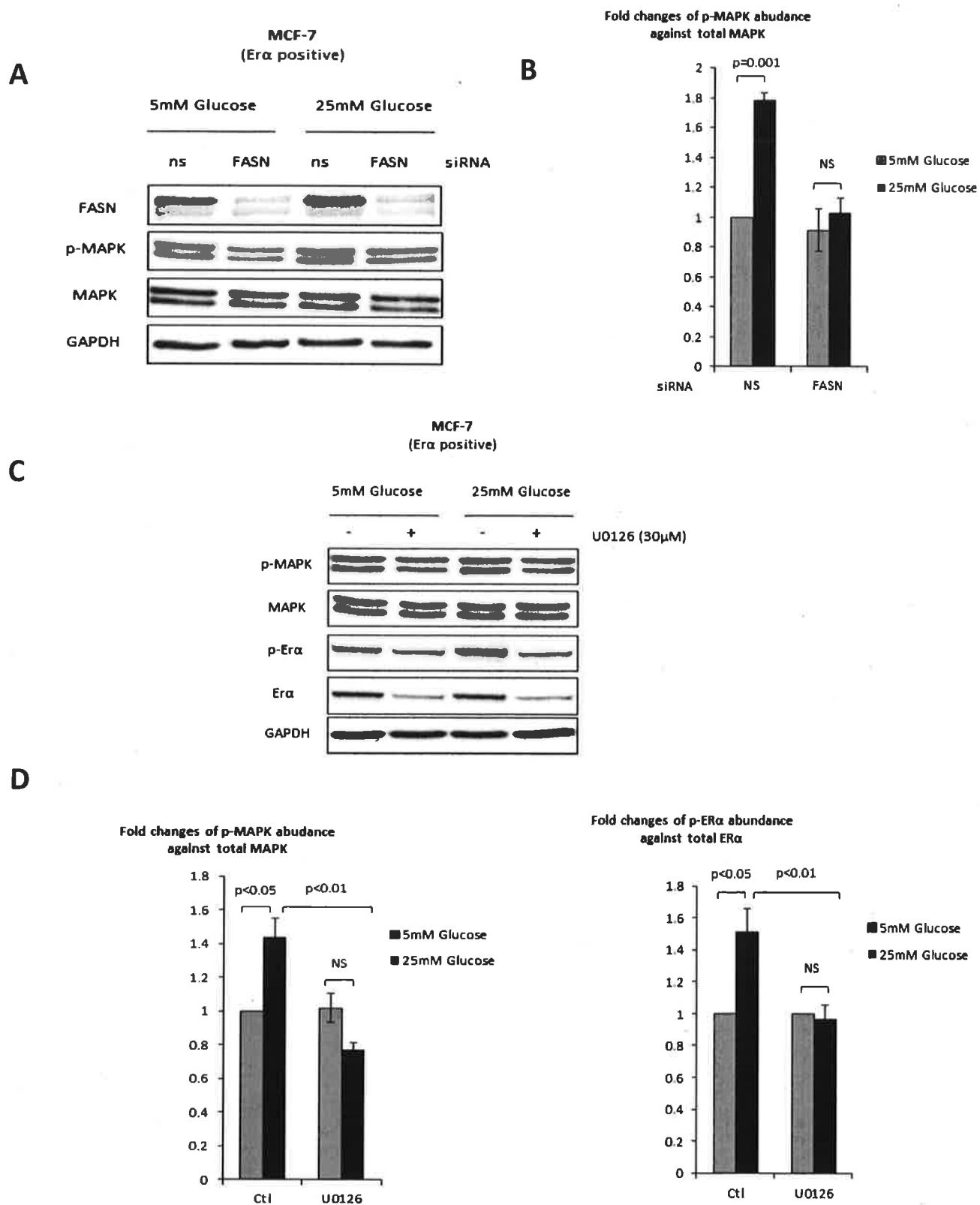


Figure 6

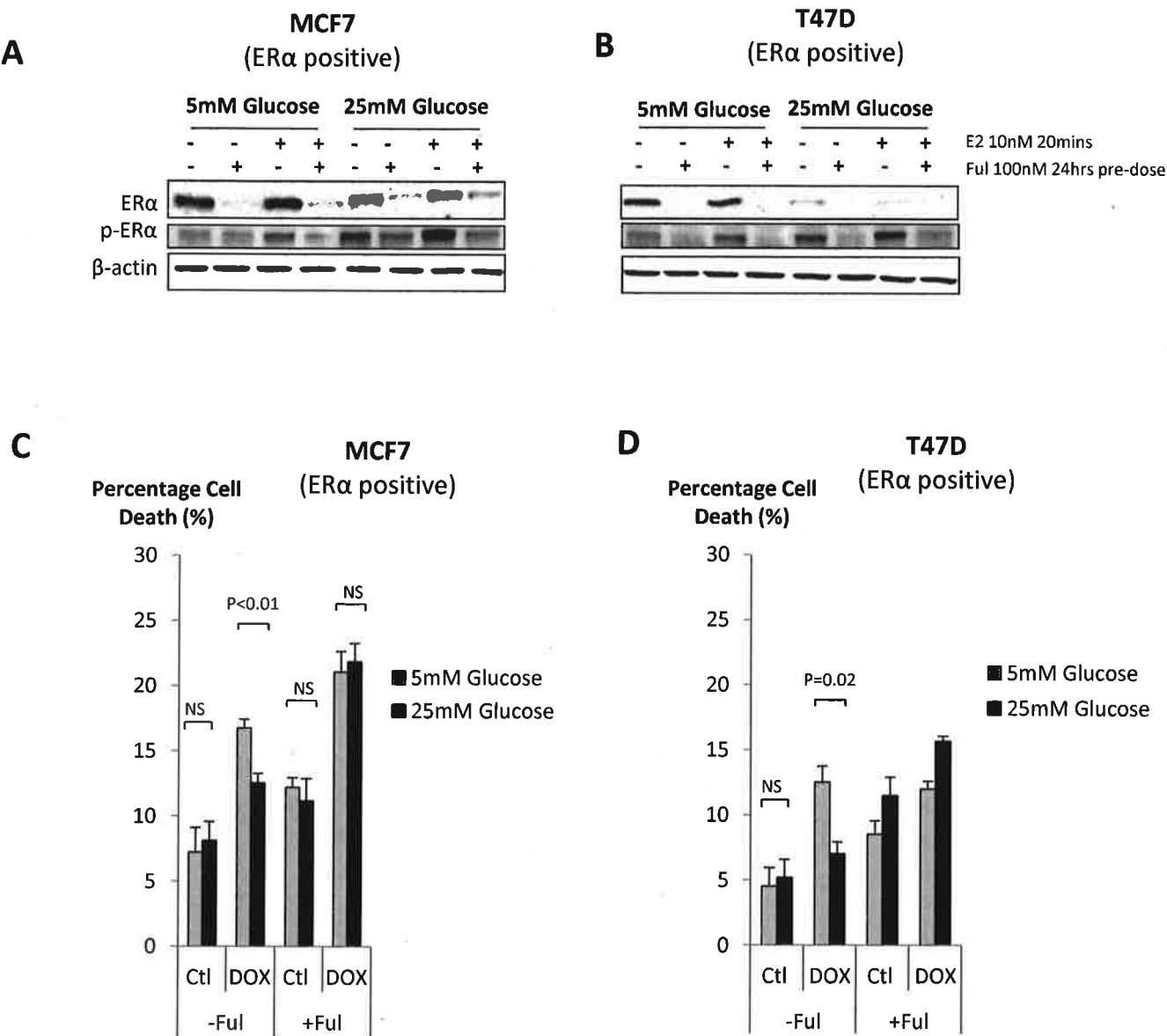
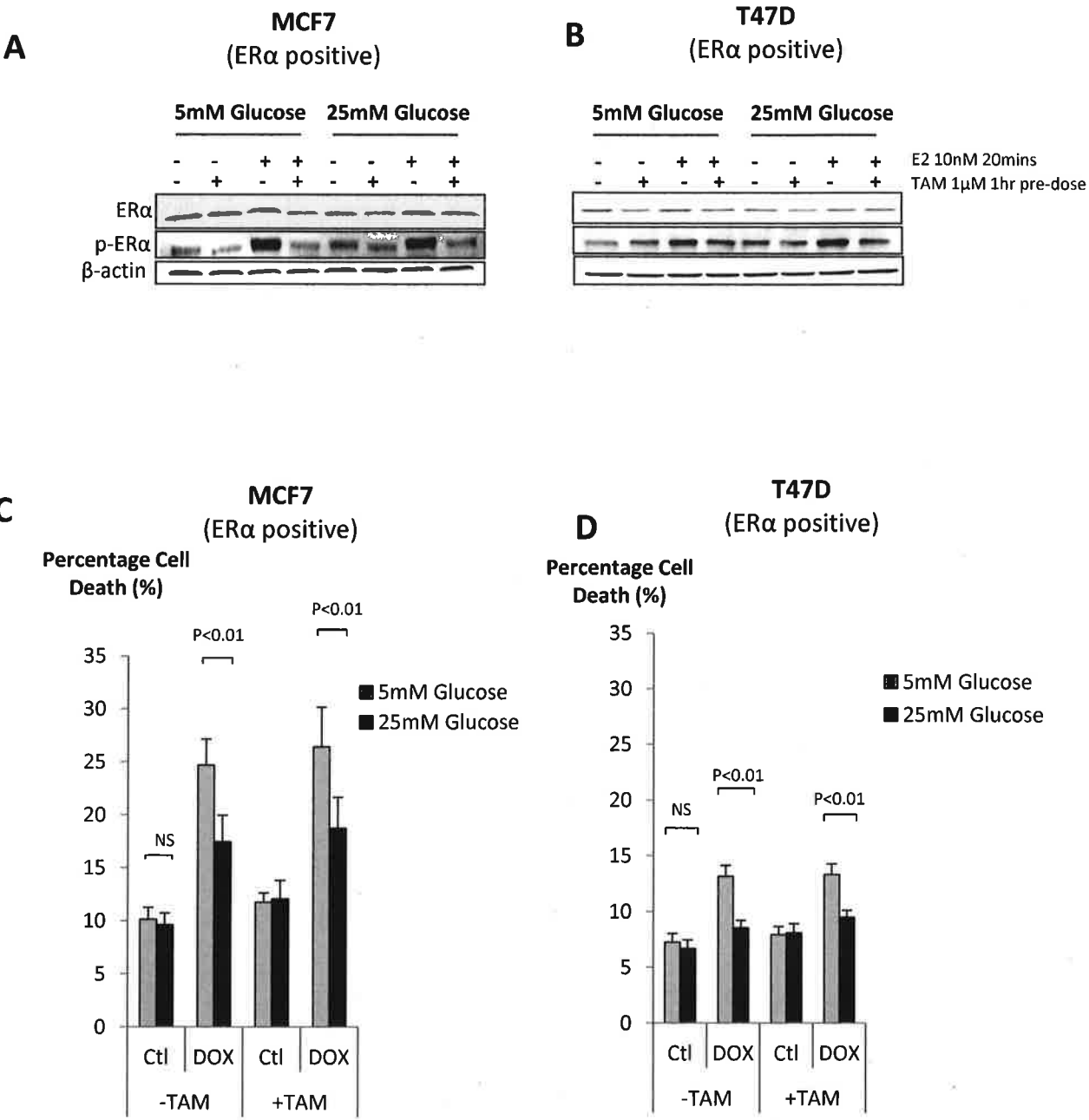
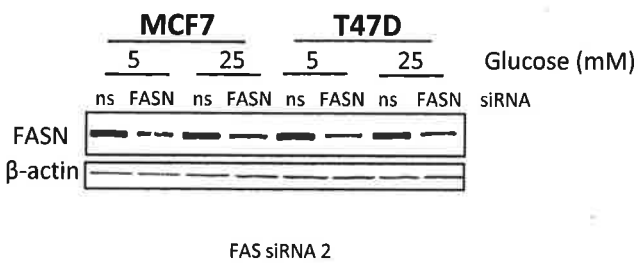


Figure 7

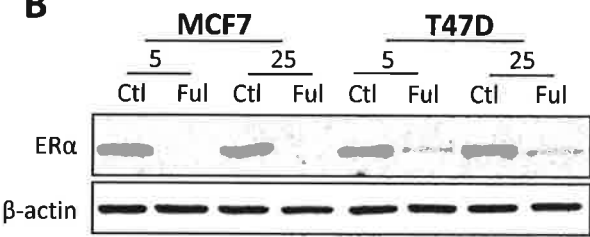


Supplementary Figure 1

A



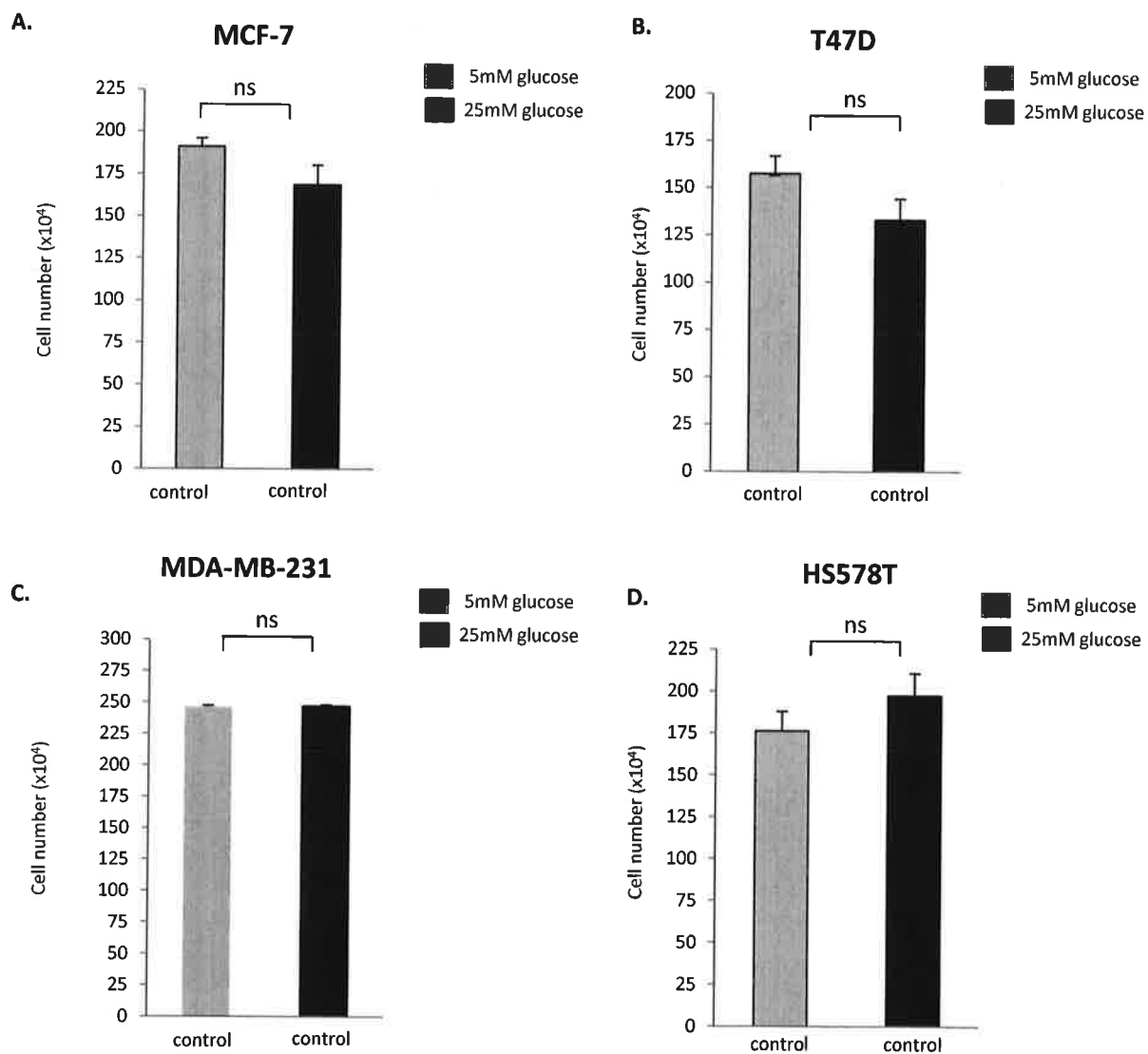
B



C

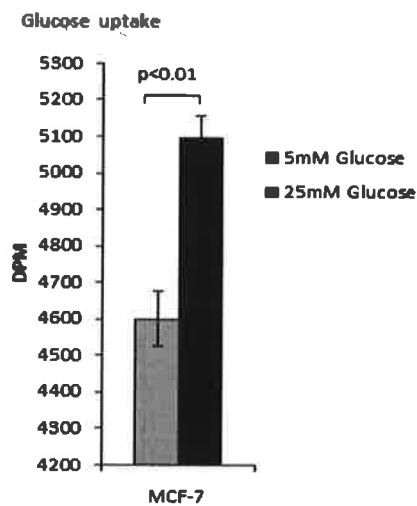


Supplementary Figure 2

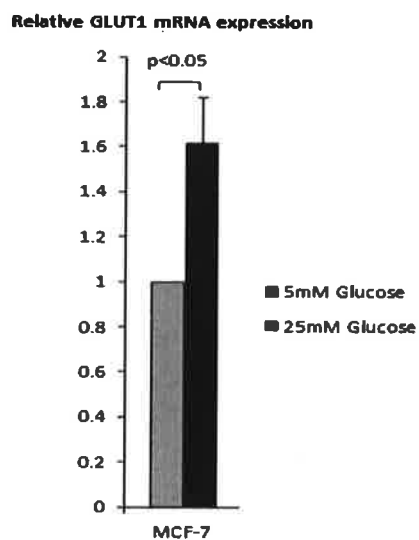


Supplementary Figure 3

A



B



C

